Supplemental Material

Mitochondrial Oxidative Stress Significantly Influences Atherogenic Risk and Cytokine Induced Oxidant Production

Corey M Harrison[1], Melissa Pompilius[1], Kent E. Pinkerton[2] and Scott W. Ballinger[1,3]

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Methods Supplement:

Generation of apoE -/-, SOD2 +/- mice: We have generated maintained the apoE -/-, SOD2 +/- mouse line at the University of Alabama at Birmingham for over 40 generations. Consequently, animals used for these studies were at least 40 generations or more from the original founders of the apoE -/-, SOD2 +/- line. These mice were originally generated by crossing apoE -/- (Jackson Laboratory **stock #002052** C57BL/6J background) with the SOD2 -/+ animal (Jackson Laboratories **stock #002973** C57BL/6J background) The apoE $^{-/-}$ mouse lacks apolipoprotein E, a ligand for lipoprotein receptors, and has elevated levels of serum VLDL, LDL cholesterol and triglycerides, developing atherosclerotic plaques similar to humans (Zhang et al. 1992).The SOD2 $^{+/-}$ mouse is deficient in the mitochondrial form of SOD2. Homozygous null (SOD2 $^{-/-}$) mice survive less than 2 weeks from birth, whereas the heterozygotes are phenotypically indistinguishable from the wild-type, but have decreased SOD2 protein levels and activity(Lebovitz et al. 1996). Studies have shown that there is not a significant induction of CuZnSOD in SOD2 mice ($^{+/-}$ or $^{-/-}$)(Van Remmen et al. 1999). Male animals used for these studies were siblings and were descendant of at least 40 generations from the original apoE $^{-/-}$, SOD2 $^{+/-}$ founders. All experimental endpoints utilized a N = 5 mice/genotype/exposure group.

The approach for the generation of the apoE -/-, SOD2 -/+ is outlined below:

F_o apoE -/- X SOD2 -/+ mating of apoE knockout with SOD2 heterozygotes

F_1 50% apoE -/+, SOD2 -/+; F_1= double heterozygotes ;

Backcross F1 double heterozygotes with apoE -/-

F_2 25% apoE -/-, SOD2 -/+ Desired apoE -/-, SOD2 +/- (used as founders)

apoE -/-, SOD2 +/- animals are utilized for generating litters; consequently, both apoE -/-, SOD2 +/+ (control siblings) and apoE -/-, SOD2 +/- mice were generated.

Diets: Mice were fed chow diets (PicoLab Rodent Chow 20) that contain 4.5% fat by weight (0.02% cholesterol). Diet and water were supplied *ad libitum*. Animals were put on respective diets at 4 weeks of age and fed until sacrificed.

apoE genotyping: Genotyping employs multiplex PCR, using primers oIMR 180 (5'-GCCTAGCCGAGGG AGAGCCG-3'), oIMR 181 (5'-TGTGACTTGGGAGCTCTGCAGC-3'), and oIMR 182 (5'-GCCGCCCCGACT GCATCT-3'). Primer pairs 180/181 yield a 245 bp product if the wild-type allele is present, whereas pair 180/182 yield a 155 bp product if the knockout gene is present.

SOD2 genotyping: Mouse genotypes were determined by PCR analysis of genomic DNA extracted (Qiagen miniprep) from tail clips. The SOD2 knockout allele is screened by using PCR primers SOD2a (5'-CAGCCCTGGCGTCGTGATTAGT-3') and SOD2b (5'-CGTGGGGTCCTTTTCACCAGCAA-3'), yielding a 2.3 kb product; a second, different primer set is used as a confimatory reaction, using primers SOD2c (5'-GAGCGACCTGA GTTGTAACATCTCC-3') and SOD2d (5'-GACCCCACGAAGTGTTGGATATAAG-3') yielding a 2.1 kb PCR product. Because SOD2 $^{-/-}$ mice die prior to weaning, genotypic distinction of SOD2 $^{+/-}$ from the SOD2 $^{-/-}$ mice is not required. apoE $^{-/-}$ genotyping employs a multiplex PCR reaction using primers hprt-a (5'-GCCTAGCCGAGGG AGAGCCG-3'), hprt-b (5'-TGTGACTTGGGAGCTCTGCAGC-3'), and hprt-c (5'-GCCGCCCCGACT GCATCT-3'). Primer pairs hprt-a/hprt-b yield a 245 bp product if the wild-type allele is present, whereas pair hprt-a/hprt-c yield a 155 bp product if the knockout gene is present.

ETS exposure: Exposures were conducted at the inhalation facilities of Dr. Kent Pinkerton (Institute of Toxicology and Environmental Health, University of California, Davis). We used a side-stream ETS dose of 10 mg/m^3 TSP. This dose of ETS is well within, or below published reports on ETS exposure in rodents. Commencing at 6 weeks of age, apoE $^{-/-}$ and sibling apoE $^{-/-}$, SOD2 $^{+/-}$ mice were exposed to either filtered air or ETS (10 mg/m^3 total suspended particulate (TSP)) for 6 hrs/day for 5 days a week for 4 weeks. The average levels of TSP, nicotine and carbon monoxide were 10.06 + 1.11 mg/m^3, 1.40 + 0.49 mg/m^3 and 32.62 + 5.56 ppm, respectively, for the entire exposure period of 4 weeks. This dose of cigarette smoke is well below that of similar studies in rodents, (Witschi et al. 1997) and falls between that of passive and active cigarette smoke exposure in humans (TSP 1-40 mg/m^3, nicotine: 1-3.3 mg/m^3; CO: 2.5-23 ppm (Witschi et al. 1997) analogous to cigarette smoke exposure within an automobile. Blood cotinine levels in adult mice exposed to this regimen were between 37 – 61 ng/ml, which are lower than those reported for other rodent models, and urine and blood cotinine levels observed in human smokers, and slightly higher than those observed in children exposed to parental smoking (Alberg et al. 2000). Similarly, typical carboxyhemoglobin (COHb) levels in human smokers are ~6% (Alberg et al. 2000); COHb levels in apoE $^{-/-}$ mice have been previously reported to be ~10% using an ETS dose 5 times higher than the level used in this study (Alberg et al. 2000). Consequently, the exposure regimens within this study are within physiologically relevant exposures experienced by humans. Control apoE $^{-/-}$ and apoE $^{-/-}$, SOD2 $^{+/-}$ mice were exposed to filtered air only.

Smoking System: The smoke generation system is composed of four major components: the first component automatically loads, lights, and smokes cigarettes, the second component is a dilution chimney, which collects and transfers smoke from the burning end of the smoldering cigarette to the third component, the conditioning chamber. The conditioning chamber is used to dilute and mix the smoke with filtered air until the desired concentration is achieved. Together, these three components constitute the smoke generation portion of the system. The final component of the system consists of chambers for the exposure of animals to aged and diluted side-stream smoke. Chamber levels of carbon monoxide, nicotine, and TSP will be monitored daily.

Research Cigarettes: The 1R4F cigarette, a standard reference cigarette was used. These cigarettes were formulated and manufactured in 1983 by the Tobacco and Health Research Institute (University of Kentucky, Lexington, KY). All cigarettes were stored at 4^{o}C until needed. At least 48 hours prior to use, cigarettes were placed in a closed chamber at 23^{o}C along with a solution of glycerin/water (mixed in a ratio of 0.76/0.26) to establish a relative humidity of 60%.

Sacrifice: Animals were weighed following paralysis by intraperitoneal injection of Ketaset/Xylazine (60 mg/kg Ketamine/10 mg/kg Xylazine). For enzymological and histological analyses, aortas were used immediately after removal. Separate animals (N=5 per group) were used for the oil red-O and enzymological analyses on aortic tissues. Otherwise, tissues (heart, aorta, brain, liver, lung, buffy coat, and plasma) were be immediately removed and frozen in liquid nitrogen, and stored at -80^{o}C until use.

Oil-red-O staining of whole aortas: Whole hearts with attached aortas were dissected free, rinsed in PBS, fixed in 10% phosphate buffered formalin, and stored in a solution of PBS + 0.1% NaN_3 at 4^{o}C. Using a dissecting microscope (Zeiss Stemi 2000 Stereomicroscope), hearts and excess fatty tissue (outside of the aorta) were removed, and the aorta cut longitudinally to allow visualization of the interior walls (lumen). Aortas were placed in oil-red-O stain at room temperature with constant agitation. Oil red-O is an oil-soluble dye, which partitions into lipids and stains neutral fats a brilliant red color. 60% isopropanol was used as the dye solvent since it causes minimal lipid removal. Stained aortas were dissected free of extraneous tissues, placed on a dry slide and flattened using a microscope coverslip and Aquamount^{TM} (Lerner Laboratories) as *en face* mounts, and photographed (Nikon 995 digital camera on Zeiss dissecting stereoscope). Atherosclerotic lesions were analyzed using Metamorph (Molecular Devices) by adjusting threshold for the red channel to select only lesion areas in the majority of the samples and using the same numerical threshold in all samples for quantification. Total lesion areas were calculated for each aorta as well as the area of the whole aorta itself. A ratio of aortic lesion area vs. total area was obtained.

Quantitative PCR (QPCR) for evaluating DNA damage: QPCR assesses generalized DNA damage in a gene-specific manner that does not require large amounts of DNA. The principle of this gene-specific assay is that DNA lesions will block rTth polymerase and therefore will lead to a decrease in amplification. Sensitivity of the assay is increased through amplification of large targets (thereby increasing the probability of encountering a DNA lesion). We have used this assay to accurately quantify DNA damage both *in vitro* and *in vivo*. A 16,059 bp QPCR product, which encompasses all but 236 bp of NADH5/6 genes in the mouse mtDNA genome, is amplified using primer set M13597 FOR (13597-13620 bps) and 13361 REV (13361-13337 bps). Genomic DNA is quantified fluorescently (PicoGreen, Molecular Probes) on a Cytofluor 4000 Series fluorometer, and 15 ng was used for QPCR. Copy number differences were normalized using a short QPCR reaction, which yielded products directly related to gene copy numbers using primers 13597F/13713R

(5'CCCAGCTACTACCATCTTCAAGT/GATGGTTTGGGAGAT TGGTTGATGT3') for the mtDNA.

DNA damage was quantified by comparing the relative efficiency of amplification of large (>15 kb) fragments of DNA and normalized this to gene copy numbers by the amplification of smaller (<250 bp) fragments, which have a statistically negligible likelihood of containing damaged bases. Hence, to measure mtDNA damage, long (*L*) and short (*S*) QPCRs were performed to determine DNA damage and number of gene copies present, respectively. To calculate lesion frequencies, the long QPCR values (*L*) were divided by the corresponding short QPCR results (*S*) to account for potential copy number differences between samples (*L/S).* Normalized values from damaged ($L_d/S_d = A_d$) samples were compared to non-damaged controls ($L_o/S_o = A_o$) resulting in a relative amplification ratio (A_d/A_o). Assuming a random distribution of lesions and using the Poisson equation [$f(x) = e^{\lambda}\lambda^x/x!$, where λ = the average lesion frequency] for the non-damaged templates (zero class; $x = 0$), the average lesion frequency per DNA strand was determined; $\lambda = -ln\ A_d/A_o$.

Western Analysis and protein nitration. Antibodies for SOD2 and 3-nitrotyrosine are commercially available (Research Diagnostics Inc. and Calbiochem, respectively). To perform immunoblots, aortic homogenates were prepared and 25 □g of the protein was loaded onto 12% SDS-PAGE gels, subjected to electrophoresis and immunoblotted with the desired antibody. To detect protein carbonyls (oxidation), homogenates were treated with 10mM 2,4-dinitrophenylhydrazine (DNPH), dissolved in 20mM Tris-HCL buffer and subjected to electrophoresis. Immunoblots were performed with anti-DNP antibodies (Sigma). Blots are visualized using chemiluminescence of the secondary HRP-goat anti-rabbit IgG.

SOD activity: Aortic homogenates were prepared and 10 □g of the homogenate assessed for
SOD activity using the cytochrome C reduction assay. This assay is based upon the ability of SOD to inhibit the reduction of cytochrome C by $O_2^{\cdot-}$ generated by xanthine/xanthine oxidase. Increased SOD activity results in inhibition of cytochrome C reduction, reflected by decreased absorbance at 550 nm. If mitochondrial isolation is not desired, cyanide and azide (3 mM KCN, 3mM NaN_3) is used to inhibit SOD1 and SOD3, allowing for direct measurement of SOD2 activity.

Cholesterol determination: Plasma samples were collected and analyzed using the cholesterol lipoprotein profile (CLiP) apparatus. The apparatus consists of two biocompatible HPLC pumps (Model 2150, Pharmacia, Piscataway, NJ), an injector (Model 9125, Rheodyne, Cotati, CA) with a 200-µL sample loop, a heating block (TC-50 controller and FH-40 reactor, Eppendorf, Brinkmann Instruments Inc., Westbury, NY) containing 15 m of Teflon tubing (1/16" o.d., 0.02" i.d.), a 30 x 1 cm Superose 6 column (Pharmacia), and a spectrophotometric detector reading at 500 nm, usually at a sensitivity setting of 0.1 (Model UA-5 with 0080-012 10 µm flow cell, Isco, Lincoln, NE). The running buffer was 0.9% NaCl/2 mM EDTA/0.01% sodium azide (pH = 7.4). Plasma samples (5 or 10 µL) were injected onto the Superose 6 column at a flow rate of 0.4

mL/min. Injections were done using a 25-µL syringe (#1702, Hamilton Co., Reno, NV). Immediately after the column, cholesterol reagent (8 g Cholesterol 1000 [Sigma Diagnostics; St. Louis, MO] and 5 mL BRIJ-35 [Sigma] brought to a total volume of 100 mL with distilled water, at a flow rate of 0.2 mL/min) was mixed with the column eluent through a low-dead-volume mixing tee, and the mixture entered the heating block (set at 55°C). Outflow from the heating block entered the spectrophotometric detector, and the cholesterol profile was collected into a computer at the rate of four data points per min. A back-pressure regulator (40 psi, Upchurch, Apple Valley, MN) was required in the outflow line from the detector to avoid air bubble formation. Both running buffer and cholesterol reagent were degassed thoroughly to further minimize bubble formation in the detector. Profiles were decomposed into component peaks and analyzed for relative area using PeakFit (SPSS Science, Chicago, IL), and absolute cholesterol values for total cholesterol and each component peak (HDL, LDL, and VLDL) were determined by comparison with a control samples of known values.

Amplex Red: H_2O_2 generation was calibrated by constructing standard curves using known H_2O_2 concentrations. Amplex Red was obtained from Molecular Probes (Eugene, OR). Aortic homogenates were diluted to a final volume of 50□l. 50 µl of the standard curve samples, controls, and experimental samples were added to individual wells of a microplate. 50 µl of the Amplex Red reagent/HRP working solution was added to each microplate well containing the standards, controls, and samples. Microplates were incubated at room temperature for 30 minutes, protected from light. Fluorescence emission was measured at ~590 nm. For each point calculated, the no- H_2O_2 control was subtracted from the value derived to correct for background fluorescence.

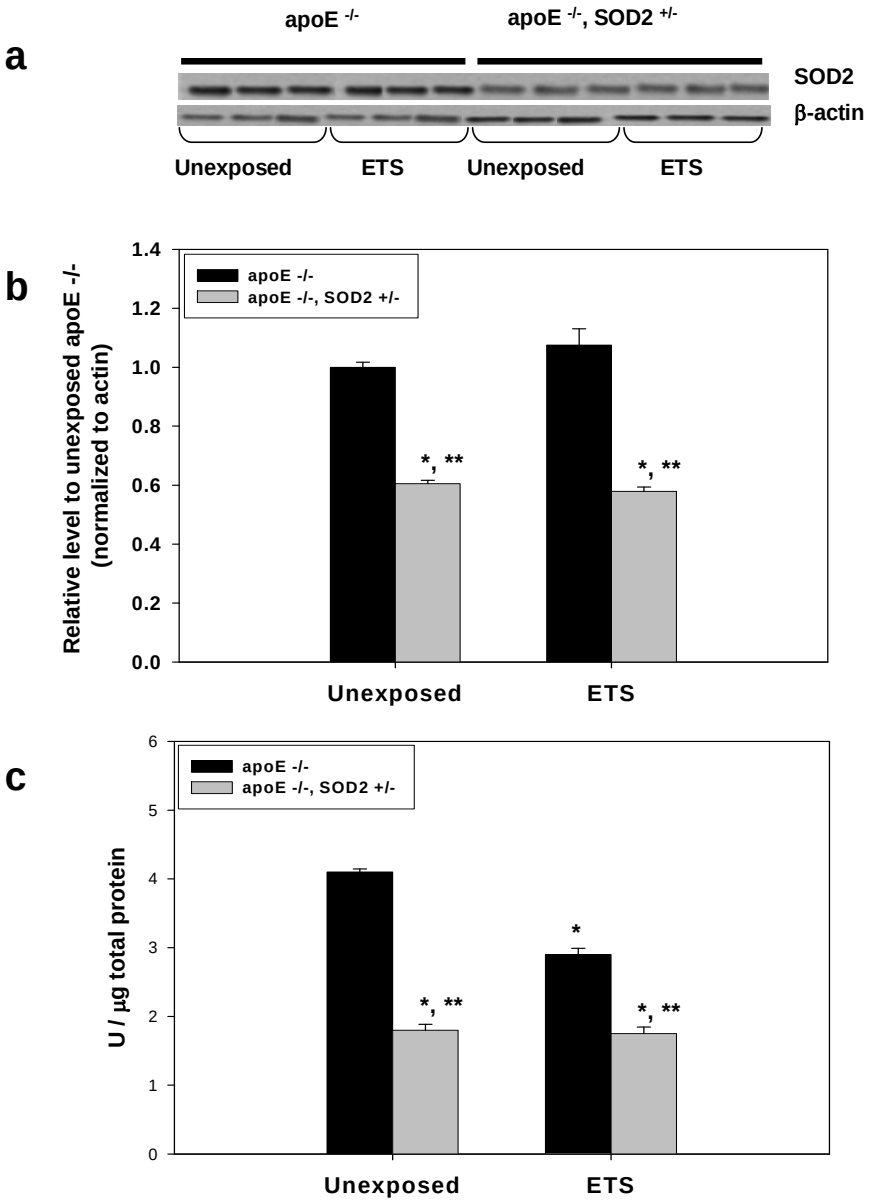
TNF-α ELISA: Aortic homogenate levels of TNF-*α* were measured using the Mouse TNF-α ELISA Kit (Endogen) according to manufacturer protocol. Diluted samples were added to the anti-mouse TNF- *α* precoated plates. Plates were then washed and incubated with a biotinylated antibody reagent. After three more washes, plates were then incubated with streptavidin-HRP concentrate. After 1 hour, the absorbance was measured at 450nm and TNF-α levels were calculated in pg/ml, then normalized to µg of total protein per sample.

siRNA Transfection: Silencer small interfering (si)RNA was custom designed for Mn-SOD (SOD2) by Qiagen, Germany. HUVECs were treated with siRNA negative control or siRNA oligonucleotides specific for human MnSOD (Hs_SOD2_5 HP). The siRNA/lipofectamine complex was generated by adding 6 □l of lipofectamine 2000 into 750 □l of Optimem and incubated at room temp for 5 min. siRNA was added to the solution to give a final concentration of 40 □M siRNA. Confluent HUVEC monolayers from passages 3 to 5 were treated with siRNA for MnSOD or siRNA control (N=6 per treatment condition) for 24hrs, followed by treatment with 30ng/ml of TNF-alpha for 30 min. Cells were then washed and visualized using fluorescence microscopy or lysed for further experiments.

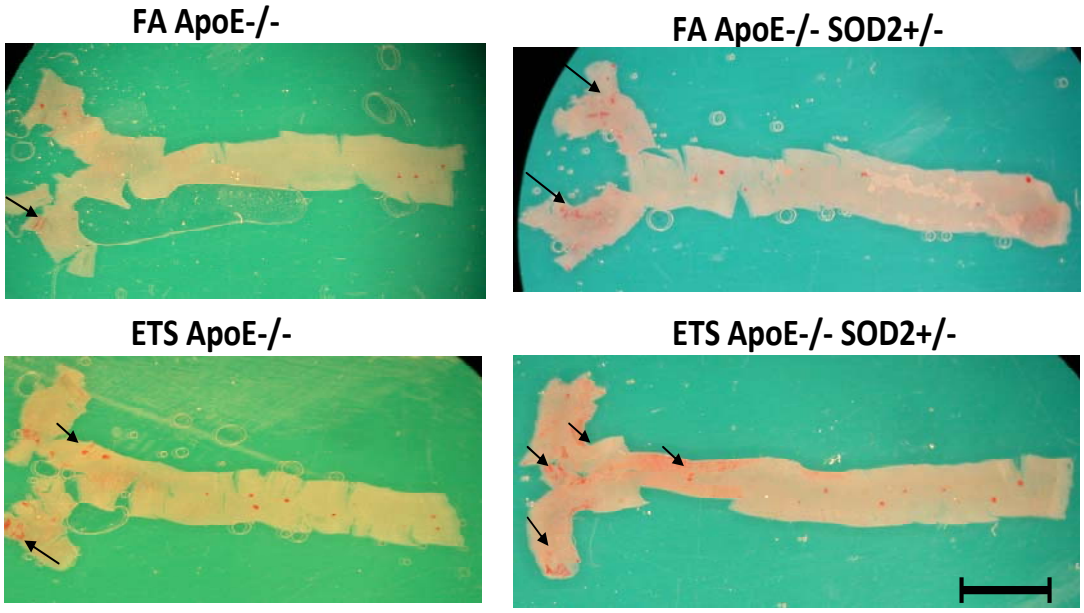
DHR fluorescence: A stock solution of 10 mM dihydrorhodamine 123 (DHR123, Molecular Probes; Eugene, OR) was prepared in 100% dimethyl sulfoxide and further

diluted to 20 □M in serum free MEM media. HUVECs were treated with siRNA and TNF-alpha for 24 hours and 30 min respectively. HUVECs subsequently treated with DHR123 at a final concentration of 10 □M were incubated for 30 min at 37°C in 5% CO_2 and were briefly rinsed three times with Earle's balanced salt solution (EBSS). Cells were immediately analyzed with a 25X or 40X objective lens in a Nikon Eclipse TE2000-U microscope with epi-illumination. The excitation wavelength was 485 nm and emission wavelength of 530 nm. The cell preparations in each experiment were photographed at the same light intensity and time of exposure. For quantitative studies, the intensity of fluorescence was calculated using Image J software per instructions in the software manual.

Aconitase Activity: Aconitase is specifically inactivated by superoxide ($O_2^{\bullet -}$) and peroxynitrite ($ONOO^-$), and thus can serve as an indirect indicator of oxidant stress related to these molecules. Aconitase activity was determined in aortic mitochondrial isolates (2 aortas/ isolate) by measurement of the formation of NADPH from NADP at 340 nm in the presence of citrate and $NADP^+$-dependent isocitrate dehydrogenase (2 U/ml) in 50 mM Tris·HCl (pH 7.4), 30 mM sodium citrate, 0.5 mM $MnCl_2$, and 0.2 mM $NADP^+$ at 25°C.



Supplemental Figure 1: **SOD2 protein levels and activity in apoE-/- and apoE-/-, SOD2+/- mice exposed to filtered air or ETS.** Mice were exposed to either filtered air (Unexposed) or 10 mg/m3 total suspended particulate of ETS (ETS) for 4 weeks and SOD2 protein and activity were measured from aortic tissue homogenates. **(a)** Immunoblot using SOD2 and b-actin antibodies. **(b)** Bar graph of quantified SOD2 protein, normalized to b-actin levels. **(c)** SOD2 activity. Each exposure group consisted of 5 mice/genotype/group. Asterisks (*, **) indicate significant difference ($P < 0.05$) from unexposed and exposed apoE -/- group, respectively.



Supplemental Figure 2. **Atherosclerotic lesion (aortic oil red-O staining) assessment in apoE-/- and apoE-/-, SOD2+/- mice exposed to filtered air or ETS.** Mice were exposed to either filtered air (Unexposed) or 10 mg/m3 total suspended particulate of ETS (ETS) for 4 weeks and aortas were extracted, dissected, and stained with oil-red-O at room temperature with constant agitation. Scale bar for photomicrgraphs is 3mm.

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